

Original Article

Baicalein induced apoptosis and autophagy of undifferentiated thyroid cancer cells by the ERK/PI3K/Akt pathway

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Abstract: Thyroid cancer is the most common endocrine system malignancy, and undifferentiated thyroid cancer is one of the most invasive tumors. Studies have found that baicalein, a major flavonoid separated from the root of *Scutellaria baicalensis* Georgi, has an inhibitory effect on a variety of malignant tumor cells. However, the effect of baicalein on undifferentiated thyroid cancer has not yet been investigated. In the present study, follicular undifferentiated thyroid cancer cells (FRO) were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for 12 h, 24 h, 36 h, or 48 h; then, the cell viability and clonogenicity were measured. Cell cycles and cell apoptosis were measured by flow cytometer after FRO cells were treated with baicalein for 36 h or 48 h. After FRO cells were treated with baicalein for 48 h, the expression of apoptosis-related proteins (Bcl-2, Bax, Caspase-3 and Caspase-8), autophagy-related proteins (Beclin-1, p62, Atg5 and Atg12) and the phosphorylation levels of ERK and Akt in FRO cells were measured by Western blot. The results showed that baicalein reduced the cell viability and cell colony numbers of FRO cells in a dose- and time-dependent manner. Baicalein also induced cell apoptosis and arrested the cell cycles of FRO cells. Baicalein decreased the ratio of Bcl-2/Bax but increased the expression of Caspase-3 and Caspase-8. Furthermore, baicalein induced autophagy in FRO cells. It significantly increased the expression of Beclin-1, Atg5, p62 and Atg12. Baicalein significantly decreased the ratios of p-ERK/ERK and p-Akt/Akt, indicating that it suppressed the ERK and PI3K/Akt pathways. In conclusion, baicalein could suppress the growth of undifferentiated thyroid cancer cells by inducing apoptosis and autophagy. The inhibition of the ERK and PI3K/Akt pathways may be involved in the mechanism.

Keywords: Baicalein, undifferentiated thyroid cancer, apoptosis, autophagy

Introduction

Thyroid cancer is the most common endocrine system malignancy, accounting for approximately 1% of malignancies in total [1]. Studies have shown that thyroid cancer is one of the most rapidly increasing malignant tumor types worldwide in recent years. According to statistics from the American Cancer Society, the incidence of thyroid cancer has increased from 4.9/100,000 in 1975 to 14.3/100,000 in 2009. The incidence of thyroid cancer in women increased from 6.5/100,000 to 21.4/100,000, which is three times the incidence observed in men. Thyroid cancer has become the fifth most common malignant tumor affecting women in the United States [2]. Thyroid can-

cer can be classified into differentiated thyroid cancer (papillary thyroid cancer and follicular thyroid cancer), undifferentiated thyroid carcinoma and medullary thyroid carcinoma. Among them, undifferentiated thyroid cancer is one of the most invasive tumors. The average survival time of patients with undifferentiated thyroid carcinoma is 3-5 months [3, 4]. Although studies have reported long-term survival in patients with thyroid cancer, patients with one-year survival rate of less than 5% are estimated to represent 10-20% of total patients [5, 6]. Although only 1-3% of thyroid cancer is undifferentiated thyroid cancer, this form results in 14-50% of mortality [7]. Traditional surgical resection and radioactive iodine treatment are the main treatments for thyroid cancer, but thyroid surgery-

Baicalein induced apoptosis and autophagy of thyroid cancer cells

related complications, such as injuries to the parathyroid glands, recurrent laryngeal nerve and laryngeal nerve, seriously affect the life quality of patients [8].

Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) is one of four major flavonoids separated from the root of *Scutellaria baicalensis* Georgi, a widely used Chinese traditional medicine, Huangqin. In recent years, studies have found that baicalein has an inhibitory effect on a variety of malignant tumor cells [9, 10]. Chung et al. reported that baicalein could inhibit the proliferation of human breast cancer cells and down-regulate the expression of Cyclin D1 in breast cancer cells [11]. It could also inhibit the growth of tumors in a nude mouse model of human breast cancer [11]. Himeji et al. found that baicalein has a growth-inhibiting effect on leukemia cells [12]. Various studies also found that *Scutellaria baicalensis* Georgi and its active ingredients, baicalin and baicalein, could inhibit the growth of prostate tumor cells and promote their apoptosis [9, 10, 13]. Baicalein could also significantly inhibit the growth of malignant tumors such as bladder tumors and myeloma [14, 15]. More importantly, high concentrations of baicalein do not produce significant toxic effects on normal cells, indicating that they are relatively safe. Therefore, the clinical application prospects of baicalein as an anti-tumor drug present obvious advantages over some classical drugs [16, 17].

Apoptosis is a highly conserved cell death model that plays an important role in multiple physiological and pathological processes [18]. Activation of both exogenous cytotoxic substances and endogenous cellular signaling pathways activates the apoptotic pathway. The endogenous mitochondrial pathway is the central target of the apoptotic pathway. Studies found that Bcl-2-related proteins are the most important proteins that regulate apoptosis. The primary role of Bcl-2 is to inhibit apoptosis. Activation of Bcl-2 can promote cell growth and resist cell death, resulting in abnormal increases in cell number and tumor growth. Bax, which is highly homologous to Bcl-2, could promote apoptosis. As a result, the balance between Bcl-2 and Bax is the key to the occurrence of apoptosis [19].

Increasing numbers of studies have indicated changes in autophagy activity in a variety of

human tumors and demonstrated that autophagy plays a dual role in promoting and inhibiting tumor development [20]. Changes in autophagy activity may be associated with abnormal regulation of certain genes, such as PI3K/Akt. Type I PI3K and its downstream signal transduction components Akt and target of rapamycin (TOR) can inhibit autophagy, whereas phosphatase and tensin homolog deleted on chromosome ten (PTEN) could induce autophagy by negatively regulating the activity of type I PI3K. On the other hand, type III PI3K is required for the delivery of autophagic vacuoles and vacuoles to lysosomes. Beclin-1 regulates the autophagy activity and localization of other ATG proteins to autophagy precursor structures by forming complexes with type III PI3K [21]. Extracellular signal-regulated protein kinases (ERK) also demonstrated a regulatory role in autophagy and tumor growth [22, 23].

To explore the potential application of baicalein on undifferentiated thyroid carcinoma, the present study examined the effects of baicalein on the growth of undifferentiated thyroid carcinoma cells (FRO cells) in addition to its impacts on apoptosis and autophagy. The Beclin-1, Bcl-2, ERK and Akt pathways were investigated to explore the underlying mechanisms.

Materials and methods

Cells and reagents

Follicular undifferentiated thyroid cancer cells (FRO) were purchased from Shanghai Institute of Biochemistry and Cellular Biology of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. Baicalein (HPLC > 98%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), diluted with dimethyl sulfoxide (DMSO) and stored at -20°C in the dark. On the test day, it was added into the DMEM medium to the desired concentrations. The CCK-8 cell viability measurement kit and Annexin V-FITC Apoptosis Detection Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit anti-Bcl-2, anti-Bax, anti-Caspase-3, anti-Caspase-8, anti-Beclin-1, anti-p62, anti-Atg5, anti-Atg12, anti-phospho-ERK, anti-ERK, anti-phospho-Akt, anti-Akt, anti-GAPDH, and HRP-linked anti-rab-

Baicalein induced apoptosis and autophagy of thyroid cancer cells

bit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell viability assay by CCK-8 method

Cells were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for 12 h, 24 h, 36 h, or 48 h, then the cell viability was measured by a CCK-8 kit according to the manual. The data are described as the mean \pm standard deviations (SD) from four independent experiments.

Clonogenicity assay

Cells were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for 12 h, 24 h, 36 h, or 48 h, then cells were trypsinized and replated in six well plates with a density of 200 cells/well to be incubated for colony formation for 12 days. Colonies were stained with 0.5% alcoholic crystal violet and then counted by a stereomicroscope (Leica, ZOOM 2000, Buffalo Grove, IL, USA).

Cell cycle measurement

Cells were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for 36 h or 48 h, then cells were digested and fixed with 70% ethanol and stained with propidium iodide. Cells in the G0/G1 phase, S phase or G2/M phase were detected on a Thermo Fisher Scientific flow cytometer (MA, USA).

Analysis of cell apoptosis

Cells were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for 36 h or 48 h. Afterwards, cells were dual stained with FITC-conjugated Annexin V and PI using an Annexin V-FITC Apoptosis Detection Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. The cell apoptosis rate was evaluated by a Thermo Fisher Scientific flow cytometer (MA, USA).

Western blot analysis

Cells were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for 48 h, then cells were lysed with cell lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 1 μ M phenylmethylsulfonyl fluoride, 1.5 μ M pepstatin A

and 0.2 μ M leupeptin. Proteins were resolved on an SDS denatured polyacrylamide gel and then transferred onto a nitrocellulose membrane, which was afterwards blocked with 5% nonfat milk. The membranes were incubated with rabbit anti-Bcl-2, anti-Bax, anti-Caspase-3, anti-Caspase-8, anti-Beclin-1, anti-p62, anti-Atg5, anti-Atg12, anti-phospho-ERK, anti-ERK, anti-phospho-Akt, anti-Akt, or anti-GAPDH antibodies overnight at 4°C. On the next day, membranes were washed and incubated with secondary antibodies and were visualized with a chemiluminescence ECL Western blotting analysis system (B&D, San Jose, CA, USA). The protein levels were quantified using ImageJ software (NIH, USA) after normalization to GAPDH.

Statistical analyses

Data are represented as the means \pm SD of four independent experiments, each performed in triplicate. The data were analyzed using SPSS 17.0 software with one-way analyses of variance (ANOVA) followed by Tukey's multiple comparison post hoc tests. $P < 0.05$ was considered statistically significant.

Results

Baicalein reduced the cell viability of FRO cells in a dose- and time-dependent manner

FRO cells were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for multiple durations (12 h, 24 h, 36 h, 48 h) before the cell viability was measured with the CCK-8 method. As shown in **Figure 1A** and **1B**, only 40 μ M and 80 μ M baicalein significantly decreased the cell viability of FRO cells when cells were treated with baicalein for 12 h or 24 h ($P < 0.05$ compared to control). As shown in **Figure 1C** and **1D**, 10 μ M, 20 μ M, 40 μ M and 80 μ M baicalein all significantly decreased the cell viability of FRO cells when cells were treated with baicalein for 36 h or 48 h ($P < 0.05$ compared to control). These results showed that baicalein decreased the cell viability in a dose- and time-dependent manner.

Baicalein decreased the cell colony numbers of FRO cells in a dose- and time-dependent manner

FRO cells were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for multiple durations (12 h, 24 h, 36 h, 48

Baicalein induced apoptosis and autophagy of thyroid cancer cells

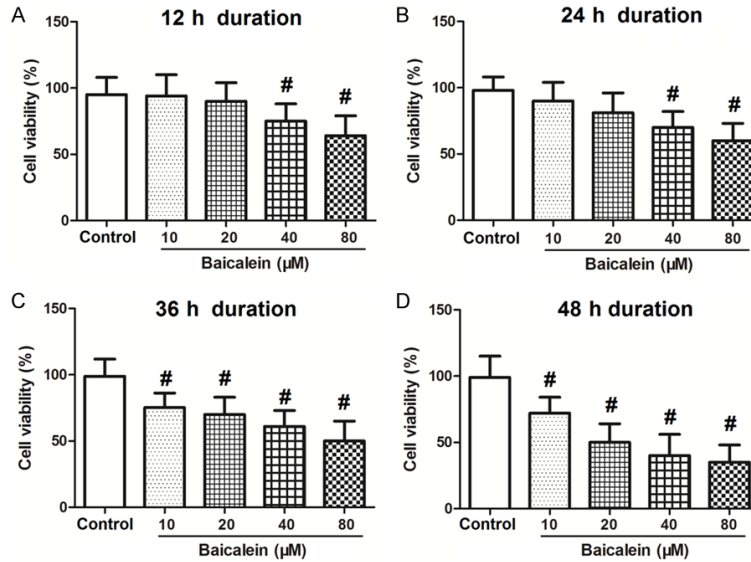


Figure 1. Effects of baicalein on the cell viability of FRO cells. FRO cells were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for multiple durations (12 h, 24 h, 36 h, 48 h) before the cell viability was measured by the CCK-8 method. Control cells were treated with DMEM medium without baicalein. (A-D) show the results after 12 h, 24 h, 36 h and 48 h, respectively. The results showed that baicalein decreased the cell viability in a dose- and time-dependent manner. Data were expressed as the mean \pm S.E.M. #: $P < 0.05$ compared to control.

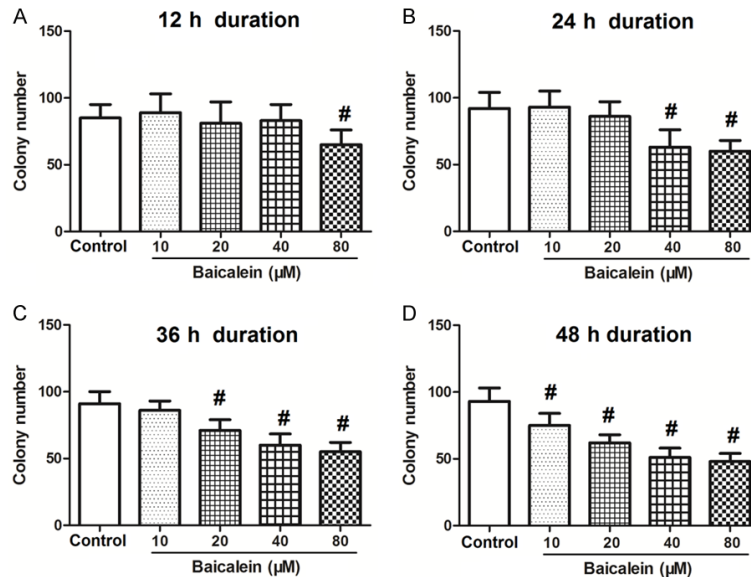


Figure 2. Effects of baicalein on the cell colony number of FRO cells. FRO cells were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for multiple durations (12 h, 24 h, 36 h, 48 h), then the numbers of cell colonies were measured. Control cells were treated with DMEM medium without baicalein. (A-D) show the results after 12 h, 24 h, 36 h and 48 h, respectively. The results suggested that baicalein decreased the cell colony numbers of FRO cells in a dose- and time-dependent manner. Data were expressed as the mean \pm S.E.M. #: $P < 0.05$ compared to control.

h), then the numbers of cell colonies were measured. As shown in **Figure 2**, only 80 μ M baicalein significantly decreased the cell colony number when cells were treated with baicalein for 12 h; 40 μ M and 80 μ M baicalein significantly decreased the cell colony number when cells were treated with baicalein for 24 h; 20 μ M, 40 μ M and 80 μ M baicalein significantly decreased the cell colony number when cells were treated with baicalein for 36 h. All dosages of baicalein significantly decreased the cell colony number of FRO cells when cells were treated with baicalein for 48 h ($P < 0.05$ compared to control). These results suggested that baicalein decreased the cell colony numbers of FRO cells in a dose- and time-dependent manner.

Baicalein affects the cell cycles of FRO cells

To measure the effect of baicalein on the cell cycles of FRO cells, the numbers of cells in G0/G1 phase, S phase and G2/M phase were calculated by a flow cytometry method after cells were treated with baicalein for 36 h or 48 h. **Figure 3** shows the representative images of cell cycles and FRO cell numbers in different cell cycles when they were treated with baicalein for 36 h or 48 h. The results demonstrate that when FRO cells were treated with 20 μ M, 40 μ M, or 80 μ M baicalein for 36 h, the cell numbers in G0/G1 phase or G2/M phase were significantly changed ($P < 0.05$ compared to control); when FRO cells were treated with 10 μ M, 20 μ M, 40 μ M, or 80 μ M baica-

Baicalein induced apoptosis and autophagy of thyroid cancer cells

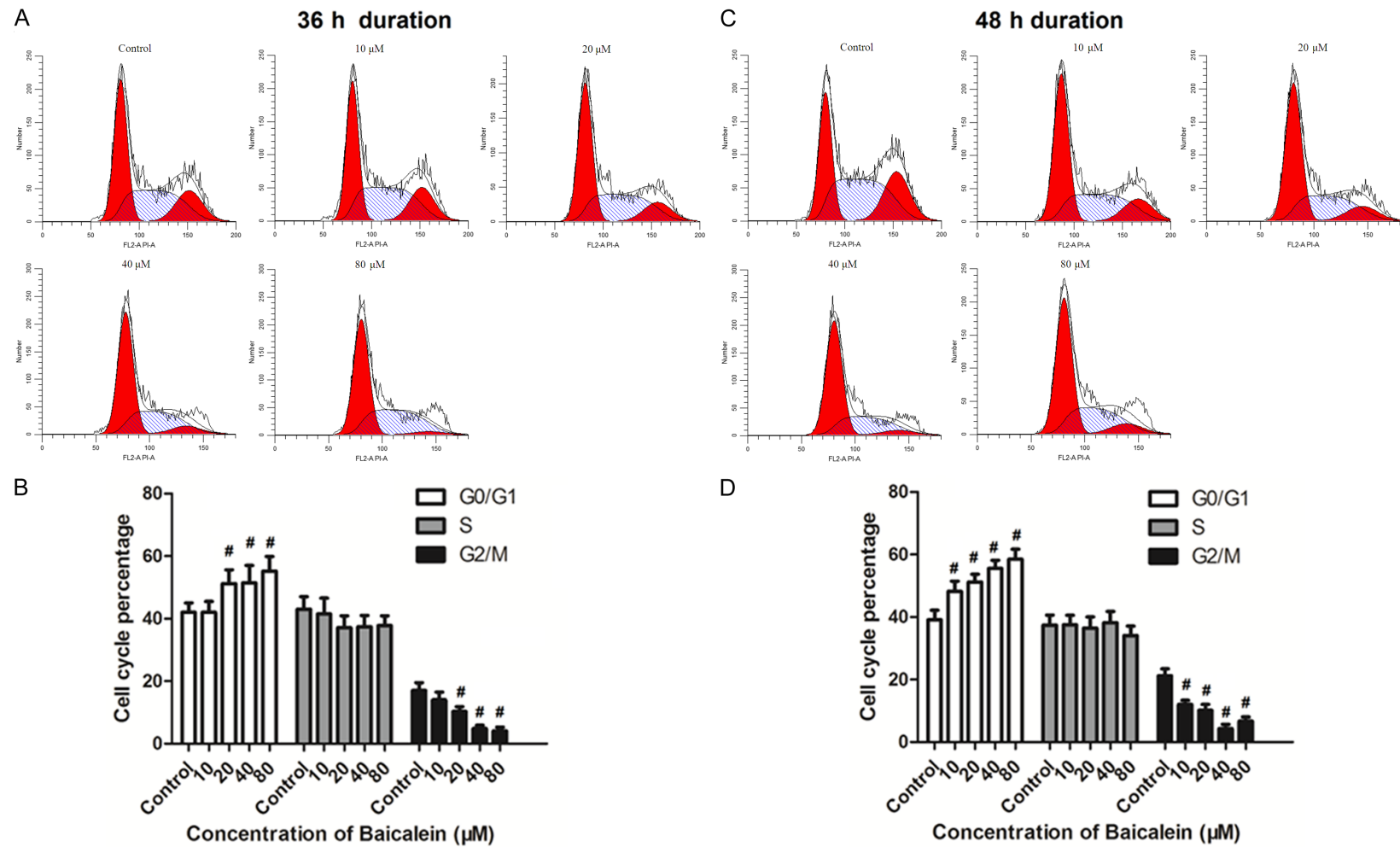


Figure 3. Effects of baicalein on the cell cycles of FRO cells. FRO cells were treated with different concentrations of baicalein (10 μ M, 20 μ M, 40 μ M, 80 μ M) for multiple durations (36 h, 48 h), then the numbers of cells in G0/G1 phase, S phase and G2/M phase were calculated by a flow cytometry method. Control cells were treated with DMEM medium without baicalein. (A and B) show the representative images of cell cycles and FRO cell numbers in different cell cycles after cells were treated with baicalein for 36 h; (C and D) show the representative images of cell cycles and FRO cell numbers in different cell cycles after cells were treated with baicalein for 48 h. Data were expressed as the mean \pm S.E.M. #: $P < 0.05$ compared to control.

Baicalein induced apoptosis and autophagy of thyroid cancer cells

lein for 48 h, the cell numbers in G0/G1 phase or G2/M phase were significantly changed ($P < 0.05$ compared to control).

Baicalein induced apoptosis of FRO cells in a dose-dependent manner

FRO cells were treated with different concentrations of baicalein (10 μM , 20 μM , 40 μM , 80 μM) for 36 h or 48 h, then the apoptotic rates were measured with the flow cytometry method. **Figure 4** shows the representative images of apoptosis and apoptotic rates of FRO cells when they were treated with baicalein for 36 h or 48 h. As shown in **Figure 4B** and **4D**, all dosages of baicalein significantly increased the apoptosis of FRO cells ($P < 0.05$ compared to control), and the effect presents a dose-dependent manner.

Effect of baicalein on expression of apoptosis-related proteins in FRO cells

To examine the effect of baicalein on the apoptosis-related proteins of FRO cells, we measured the expression levels of Bcl-2, Bax, Caspase-3 and Caspase-8 in FRO cells treated with different dosages of baicalein for 48 h by Western blotting. **Figure 5** shows the representative images of Western blot (a), the ratio of Bcl-2/Bax (b) and the fold increases of protein expression of Caspase-3 and Caspase-8 compared to control (c). The results demonstrate that 10 μM , 20 μM , 40 μM and 80 μM baicalein all significantly decreased the ratio of Bcl-2/Bax ($P < 0.05$ compared to control). Concentrations of 20 μM , 40 μM and 80 μM baicalein increased the expression of Caspase-3 and Caspase-8 ($P < 0.05$ compared to control).

Baicalein induced autophagy in FRO cells

To examine the effect of baicalein on the autophagy of FRO cells, we measured the expression of autophagy-related proteins (Beclin-1, p62, Atg5 and Atg12) in FRO cells by Western blot after they were treated with different dosages of baicalein for 48 h. **Figure 6** shows the representative images of Western blot and the fold increase of protein expression compared to control. The results demonstrate that 10 μM , 20 μM , 40 μM and 80 μM baicalein all significantly increased the expression of p62 ($P < 0.05$ compared to control), while 20 μM , 40 μM and 80 μM baicalein significantly increased the expression levels of Beclin-1,

Atg5 and Atg12 ($P < 0.05$ compared to control).

Effect of baicalein on ERK and Akt phosphorylation in FRO cells

To examine the effect of baicalein on the ERK and Akt pathways in FRO cells, we measured the phosphorylation levels of ERK and Akt in FRO cells by Western blot after they were treated with different dosages of baicalein for 48 h. **Figure 7** shows the representative images of Western blot (a) and the ratios of p-ERK/ERK and p-Akt/Akt (b). The results demonstrate that 20 μM , 40 μM and 80 μM baicalein significantly decreased the ratios of p-ERK/ERK and p-Akt/Akt ($P < 0.05$ compared to control).

Discussion

According to the International Union against Cancer (UICC)'s TNM staging and American Joint Committee on Cancer (AJCC) staging, all undifferentiated thyroid cancer should be considered as stage IV. Therapy based on combined radiotherapy, chemotherapy and surgery has been recognized and recommended worldwide. However, for radiotherapy, the toxic side-effects have become an important limiting factor. Incorrect radiotherapy may cause skin damage, esophageal toxicity damage and radiation-induced spinal cord disease. For chemotherapy, as shown in previous studies, multidrug resistance related protein (MRP) expression levels are high in undifferentiated thyroid cancer cell lines [24]. As a result, many patients with undifferentiated thyroid cancer exhibit poor prognosis after chemotherapy. Surgical resection of thyroid carcinoma, on the other hand, may injury the parathyroid glands, recurrent laryngeal nerve or laryngeal nerve [8]. Therefore, exploring new treatments for thyroid carcinoma, especially undifferentiated thyroid cancer, may significantly improve the outcome of thyroid carcinoma therapy. The present study found that baicalein reduced the cell viability and cell colony numbers of FRO cells in a dose- and time-dependent manner. Baicalein also induced cell apoptosis and arrested the cell cycles of FRO cells. Baicalein decreased the ratio of Bcl-2/Bax but increased the expression levels of Caspase-3 and Caspase-8. Furthermore, baicalein induced autophagy in FRO cells. It significantly increased the expression levels of Beclin-1, Atg5, p62 and Atg12. To examine the effect of baicalein on the ERK and

Baicalein induced apoptosis and autophagy of thyroid cancer cells

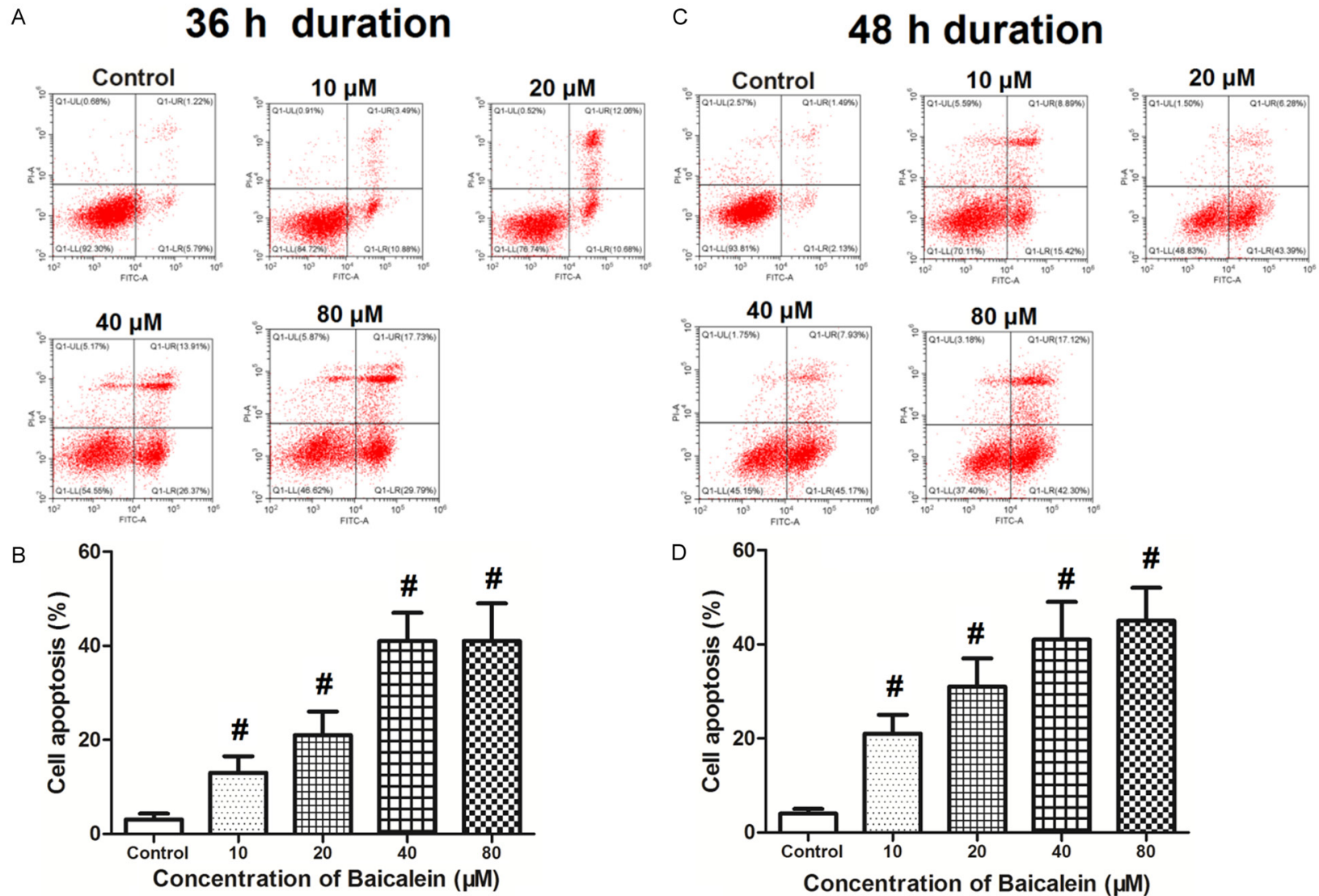


Figure 4. Effects of baicalein on apoptosis of FRO cells. FRO cells were treated with different concentrations of baicalein (10 μM, 20 μM, 40 μM, 80 μM) for multiple durations (36 h, 48 h), then the apoptotic rates were measured with the flow cytometry method. Control cells were treated with DMEM medium without baicalein. (A and B) show the representative images of apoptosis and apoptotic rates of FRO cells when they were treated with baicalein for 36 h; (C and D) show the representative images of apoptosis and apoptotic rates of FRO cells when they were treated with baicalein for 48 h. Data were expressed as the mean ± S.E.M. #: P < 0.05 compared to control.

Baicalein induced apoptosis and autophagy of thyroid cancer cells

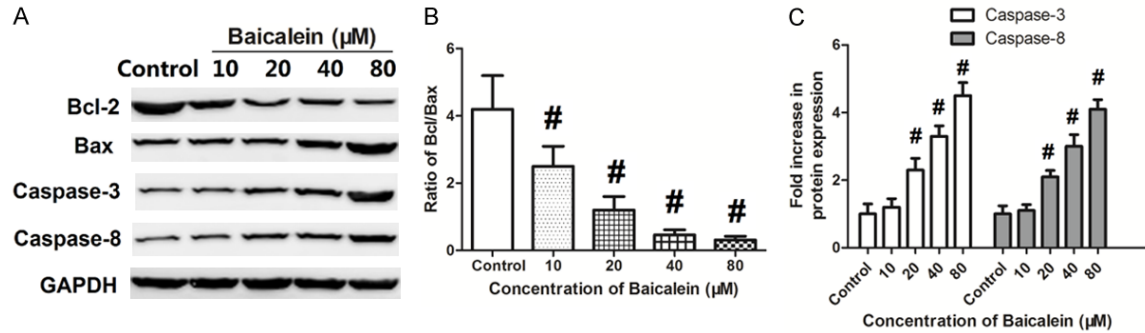


Figure 5. Effect of baicalein on expression of apoptosis-related proteins in FRO cells. The expression levels of Bcl-2, Bax, Caspase-3 and Caspase-8 in FRO cells were examined by Western blot after they were treated with different dosages of baicalein for 48 h. Control cells were treated with DMEM medium without baicalein. **Figure 5** shows the representative images of Western blot images (A), the ratio of Bcl-2/Bax (B) and the fold increase of the protein expressions of Caspase-3 and Caspase-8 compared to control (C). All concentrations of baicalein significantly decreased the ratio of Bcl-2/Bax. 20 μM, 40 μM and 80 μM baicalein increased the expression of Caspase-3 and Caspase-8. Data were expressed as the mean ± S.E.M. #: P < 0.05 compared to control.

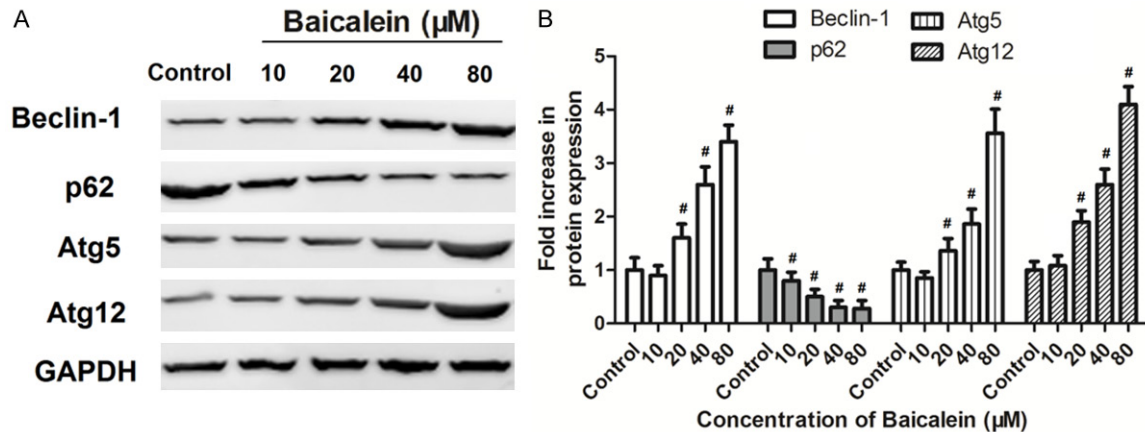


Figure 6. Effect of baicalein on autophagy proteins in FRO cells. The expression levels of autophagy-related proteins (Beclin-1, p62, Atg5 and Atg12) in FRO cells were measured by Western blot after they were treated with different dosages of baicalein for 48 h. **Figure 6** shows the representative images of Western blot images (A) and the fold increases of Beclin-1, p62, Atg5 and Atg12 compared to control (B). All concentrations of baicalein significantly increased the expression of p62; 20 μM, 40 μM and 80 μM baicalein significantly increased the expression of Beclin-1, Atg5 and Atg12. Data were expressed as the mean ± S.E.M. #: P < 0.05 compared to control.

Akt pathways in FRO cells, we measured the phosphorylation levels of ERK and Akt in FRO cells by Western blot. The results demonstrate that baicalein significantly decreased the ratios of p-ERK/ERK and p-Akt/Akt, indicating that it suppressed the activation of the ERK and PI3K/Akt pathways.

Baicalein is the main active ingredient extracted from the dried roots of *Astragalus membranaceus*, and it offers a wide range of pharmacological effects. In recent years, accumulating numbers of studies have reported the strong

anti-tumor activity of baicalein. Baicalein can induce tumor cell apoptosis, arrest cell cycles, inhibit tumor cell proliferation, and inhibit tumor neovascularization, tumor invasion and metastasis by regulating various signaling pathways [14, 22, 23, 25, 26-32, 33-35]. Baicalein could effectively inhibit the invasion and migration of various cancers, including gastric cancer [25], lung cancer [36], pancreatic cancer [27], colon cancer [37], breast cancer [38], skin cancer [39], bladder cancer [35], liver cancer [26] and osteosarcoma [34]. However, the effect of baicalein on undifferentiated thyroid cancer

Baicalein induced apoptosis and autophagy of thyroid cancer cells

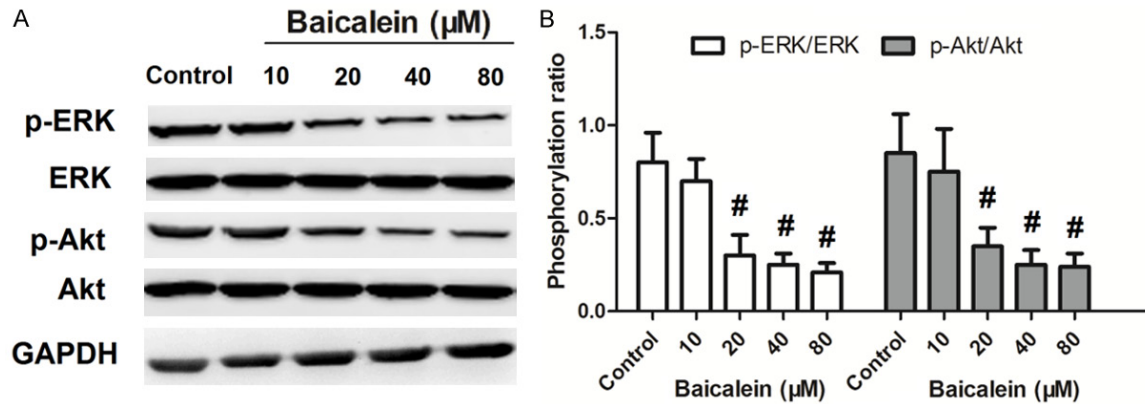


Figure 7. Effect of baicalein on ERK and Akt phosphorylation in FRO cells. The phosphorylation levels of ERK and Akt in FRO cells were measured by Western blot after they were treated with different dosages of baicalein for 48 h. **Figure 7** shows the representative images of Western blot images (A) and the ratios of p-ERK/ERK and p-Akt/Akt (B). The results showed that 20 μM, 40 μM and 80 μM baicalein significantly decreased the ratios of p-ERK/ERK and p-Akt/Akt. Data were expressed as the mean ± S.E.M. #: $P < 0.05$ compared to control.

has not been studied yet. The present study examined the cell viability of FRO cells after they were treated with different concentrations of baicalein (10 μM, 20 μM, 40 μM, 80 μM) for multiple time durations (12 h, 24 h, 36 h, 48 h). The results revealed that baicalein decreased the cell viability in a dose- and time-dependent manner. We also measured the effect of baicalein on the clonogenic growth of FRO cells with a clonogenicity assay. It was revealed that baicalein inhibited the clonogenic growth of FRO cells in a dose- and time-dependent manner. Furthermore, we calculated the number of cells in G0/G1 phase, S phase and G2/M phase by a flow cytometry method after the cells were treated with baicalein for 36 h or 48 h. The results demonstrate that after FRO cells were treated with baicalein, the cell numbers in G0/G1 phase were significantly higher than control, while the cell numbers of G2/M phase were significantly lower than control. These results revealed that baicalein could effectively arrest the cell cycles of FRO cells. Taken together, the results of cell viability, clonogenicity assay and cell cycles suggested that baicalein could effectively restrain the growth of undifferentiated thyroid cancer cells.

To investigate the mechanisms of the effects of baicalein on FRO cells, we examined its effect on the apoptosis and autophagy of FRO cells. We first examined the apoptosis rate of FRO cells with the flow cytometry method. Baicalein significantly increased the apoptosis of FRO cells, and the effect presents a dose-depen-

dent manner. Next, the expression levels of Bcl-2, Bax, Caspase-3 and Caspase-8 in FRO cells were measured by Western blot after they were treated with different dosages of baicalein for 48 h. Baicalein decreased the ratio of Bcl-2/Bax but increased the protein expression levels of Caspase-3 and Caspase-8. For autophagy, the expression of autophagy-related proteins (Beclin-1, p62, Atg5 and Atg12) in FRO cells were measured by Western blot after they were treated with different dosages of baicalein for 48 h. As shown in **Figure 6**, baicalein significantly increased the expression levels of Beclin-1, Atg5, p62 and Atg12. These results showed that baicalein could induce apoptosis and autophagy in FRO cells, which may contribute to the anti-cancer ability of baicalein. Several studies have revealed that baicalein could regulate apoptosis in cancer cells. Jiang et al. found that baicalein induced apoptosis of bladder cancer cells (T24 cells) through down-regulating miR-106, along with inhibition of the JNK and MEK/ERK pathways [30]. In another study on liver cancer cells, baicalein could promote cell apoptosis by affecting the PI3K/Akt signaling pathway together with LY294002 (an inhibitor of the PI3K/Akt pathway) [29]. In colon cancer cells, it was found that baicalein induced the apoptosis of HCT116 cells by activating mitogen-activated protein kinase (MAPK) [33]. Autophagy modulation has been considered a potential therapeutic strategy for carcinoma, but the effects of baicalein on the autophagy of cancer cells were less studied. Li et al. discovered that baicalein induced apoptosis and

Baicalein induced apoptosis and autophagy of thyroid cancer cells

autophagy and decreased P-gp and Bcl-xl expression levels in hepatocellular carcinoma cells [32]. In a study on ovarian cancer cells, baicalein was found to induce Beclin-1- and ERK-dependent autophagy [23]. Moreover, an increase was found in the phosphorylation of ERK and AKT by baicalein, and inhibition of ERK activation by the pharmacological inhibitor U0126 or ERK siRNA blocked baicalein-induced autophagy [23]. In hepatocellular carcinoma HepG2 cells, baicalein remarkably induced the formation of autophagosomes after 24-h treatment and up-regulated the expression of microtubule-associated protein 1A/1B-light chain 3-II in concentration-dependent and time-dependent manners. It also concentration-dependently and time-dependently decreased the expression levels of p-AKT, p-ULK1 and p-4EBP1, indicating the role of the AKT/mTOR pathway in baicalein-triggered autophagy [40].

To further explore the signaling pathways that baicalein may regulate in FRO cells, we examined the phosphorylation levels of ERK and Akt in FRO cells by Western blot after they were treated with different dosages of baicalein for 48 h. As shown in **Figure 7**, 20 μ M, 40 μ M and 80 μ M baicalein all significantly decreased the ratios of p-ERK/ERK and p-Akt/Akt, which suggested inhibition of ERK and Akt signaling pathways by baicalein. It has been reported that baicalein inhibited tumor invasion and migration by regulating multiple signaling pathways. Baicalein exerted its anti-metastasis activity by inhibiting the phosphorylation of MEK1 and ERK1/2. Baicalein and ERK inhibitors could synergistically inhibit tumor invasion, and the mechanism is related to the ERK pathway [22]. In another study, baicalein suppressed the expression levels of phosphorylated Akt, ERK, JNK and p38 in the MAPK/matrix metalloproteinase (MMP) signaling pathway, in addition to the expression levels of MMP-2 and MMP-9, therefore exhibiting anti-tumor metastasis effects [38]. It was also found that baicalein inhibited androgen-independent prostate cancer metastasis by down-regulating the caveolin-1/AKT/mTOR pathway [28]. Baicalein enhanced cAMP-mediated vasodilator-stimulated phosphoprotein (VASP) phosphorylation and interfered with the MAPK and PI3K/Akt signaling pathways [31]. In accordance with these studies, the present study showed that baicalein effectively down-regulated the ERK and Akt pathways. As

the ERK and Akt pathways are both closely involved in apoptosis and autophagy in cancer cells, it can be hypothesized that baicalein may induce apoptosis and autophagy in FRO cells by down-regulating the ERK and Akt pathways, thus reducing the viability and clonogenicity growth while arresting the cell cycles of undifferentiated thyroid cancer cells (FRO cells).

Conclusions

In conclusion, the present study revealed that baicalein could suppress the growth of undifferentiated thyroid cancer cells by inducing apoptosis and autophagy. The inhibition of the ERK and PI3K/Akt pathways may be involved in the mechanism of suppression.

Acknowledgements

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Disclosure of conflict of interest

None.

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Baicalein induced apoptosis and autophagy of thyroid cancer cells

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Baicalein induced apoptosis and autophagy of thyroid cancer cells

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